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United States Patent [19]

ter Huurne et al.

[11] **Patent Number:** **5,882,655**[45] **Date of Patent:** **Mar. 16, 1999**[54] **SERPULINA HYODYSENTERIAE VACCINE
COMPRISING A HYGENE MUTANT**[75] Inventors: **Agnes ter Huurne; Susie Jane Muir,**
both of Weesp, Netherlands[73] Assignee: **American Cyanamid Company,**
Madison, N.J.[21] Appl. No.: **950,433**[22] Filed: **Oct. 15, 1997****Related U.S. Application Data**[63] Continuation of Ser. No. 461,748, Jun. 5, 1995, abandoned,
which is a continuation of Ser. No. 194,127, Feb. 9, 1994,
abandoned, which is a continuation of Ser. No. 996,197,
Dec. 23, 1992, abandoned.[30] **Foreign Application Priority Data**

Dec. 23, 1991 [NL] Netherlands 91203384.2

[51] **Int. Cl.**⁶ **A61K 39/00; A61K 39/002**[52] **U.S. Cl.** **424/262.1; 424/92; 424/16;**
435/6; 435/69.3[58] **Field of Search** 424/16, 92, 262.1;
435/69.3, 6[56] **References Cited****U.S. PATENT DOCUMENTS**

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(1992) pp. 109-114.*Primary Examiner*—James C. Housel*Assistant Examiner*—Ginny Allen Portner*Attorney, Agent, or Firm*—Stevens, Davis, Miller & Mosher,
L.L.P.[57] **ABSTRACT**

According to the present invention a vaccine can be prepared containing a mutant *Serpulina hyodysenteriae* which is defective in its production of biologically active hemolysin. The mutation by which *Serpulina hyodysenteriae* is made defective in its production of hemolytically active hemolysin is established by means of genetical engineering techniques. Such mutations comprise e.g. deletion of part or the entire gene coding for hemolysin and/or nucleotide sequences controlling the production of hemolysin, or insertion of an extra nucleotide or polynucleotide into the gene encoding hemolysin and/or the nucleotide sequences controlling the production of hemolysin, or a combination of said deletion and insertion. These vaccines are useful in the prevention of *Serpulina* infections in susceptible animals such as swine.

9 Claims, 1 Drawing Sheet

FIG. 1

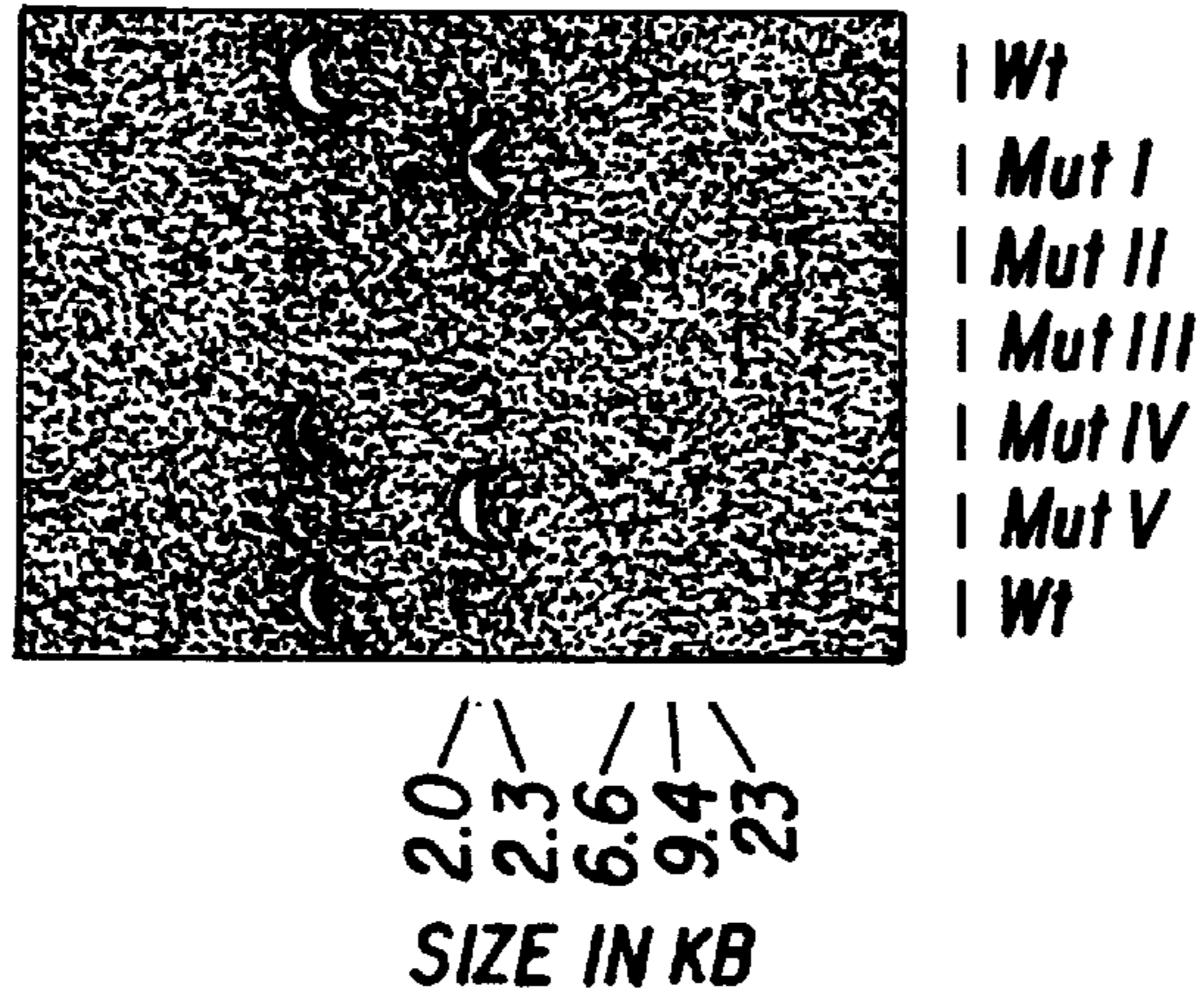
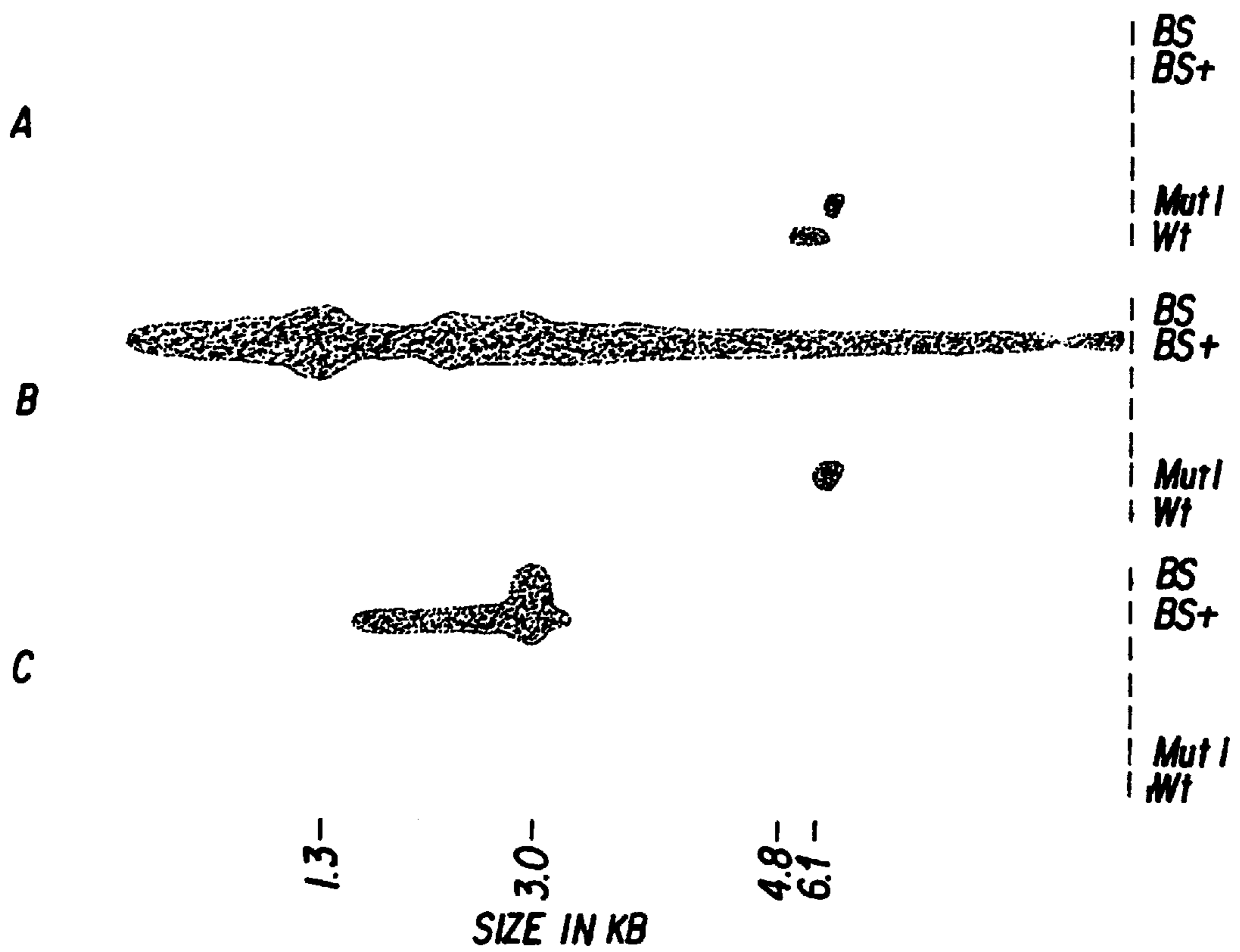


FIG. 2



**SERPULINA HYODYSENTERIAE VACCINE
COMPRISING A HYGENE MUTANT**

This application is a continuation of application Ser. No. 08/461,748, filed Jun. 5, 1995, now abandoned, which is a continuation of application Ser. No. 08/194,127, filed Feb. 9, 1994, now abandoned, which is a continuation of application Ser. No. 07/996,197, filed Dec. 23, 1992, now abandoned, which claims priority to foreign application number EP 91203384.2, filed on Dec. 23, 1991 under 35 U.S.C. 119.

The present invention is concerned with a vaccine for combating *Serpulina* (*Treponema*) *hyodysenteriae* infection and with recombinant polynucleotides and *Serpulina hyodysenteriae* mutants for the preparation of such a vaccine.

Serpulina hyodysenteriae, the major etiological agent of swine dysentery is an anaerobic, β -hemolytic spirochete found in the porcine large intestine. The disease is characterized by a mucohemorrhagic diarrhoea. This seems to be associated with the extensive superficial necrosis of the luminal epithelial lining and of the crypts of Lieberkuhn.

The disease leads to dehydration, weight loss and eventually death.

This pathogen secretes hemolysin which is thought to play an essential role in the pathogenesis of the disease.

Serpulina hyodysenteriae is differentiated from the non-pathogenic, weakly β -hemolytic *Serpulina innocens* by its hemolytic pattern on blood agar plates, or by testing enteropathogenicity in pigs or mice.

In vivo, during the acute disease course, up till now no immunogenic response induced by hemolysin could be serologically demonstrated.

Genetic approaches to elucidate the pathogenesis of spirochaetal infections have been hampered since a genetic exchange system permitting introduction of genes into spirochaetal cells was absent. No methods of transformation or general transduction have been previously described.

According to the present invention, a vaccine can be prepared containing a mutant *Serpulina hyodysenteriae* which is defective in its production of biologically active hemolysin.

The mutation by which *Serpulina hyodysenteriae* is made defective in its production of biologically active hemolysin is established by means of genetical engineering techniques. Such mutations comprise e.g. deletion of part or the entire gene encoding hemolysin and/or nucleotide sequences controlling the production of hemolysin, or insertion of an extra nucleotide or polynucleotide into the gene encoding hemolysin and/or the nucleotide sequences controlling the production of hemolysin, or a combination of said deletion and insertion. The extra polynucleotide used for said insertion may be either a natural polynucleotide fragment derived from *Serpulina hyodysenteriae* or an other organism, or an unnatural polynucleotide. The extra polynucleotide may encode a foreign protein which is expressed by the treponeme, and which might be a protein useful in the selection of the mutant and/or may be a protein characteristic for and providing immunity against *Serpulina hyodysenteriae* or an other organism. Alternatively, the extra nucleotide or polynucleotide may serve merely to cause a frame shift in the hemolysin gene, thus resulting in abolishment of the production of biologically active hemolysin.

Genetical engineering methods which can be applied in establishing a mutation in *Serpulina hyodysenteriae* that results in abolishment of hemolysin production are known in the art for analogous approaches in other organisms.

An insertion can e.g. be established by first isolating the gene encoding hemolysin of *Serpulina hyodysenteriae*,

inserting the extra nucleotide or polynucleotide into a suitable region of the coding or controlling part of said gene and transforming the *Serpulina hyodysenteriae* with said mutated gene, thereby establishing recombination of at least part of the isolated gene with the chromosome of *Serpulina hyodysenteriae*. Thereafter the *Serpulina hyodysenteriae* bacteria wherein hemolysin production is made defective are selected.

Preferably use is made of a self-replicating construct (plasmid, phage, etc.) harboring hemolysin. Prior to insertion of the extra nucleotide or polynucleotide, the gene encoding hemolysin is treated with restriction endonuclease, preferably having specificity for a restriction site which is unique in the construct. In order to be effectively ligated into the hemolysin gene, the insert should have 3' and/or 5' ends which are complementary or which are made complementary to the two ends in the hemolysin gene at the site of insertion.

Transformation of the *Serpulina hyodysenteriae* can be established by electroporation.

Genetically engineered *Serpulina hyodysenteriae* according to the present invention is useful in the prevention or combatment of *Serpulina* infections in susceptible individuals, in particular in swine. To this end use is made of a vaccine which contains and immunologically adequate amount of said genetically engineered *Serpulina* in live or inactivated form in a suitable carrier such as a buffer or the culture medium of the cells, optionally in the presence of one or more preservative constituents. In order to prepare a vaccine form which is more stable on storage, the *Serpulina* may be freeze-dried, optionally in the presence of one or more stabilizing constituents. Prior to use, the freeze-dried vaccine can be reconstituted by the addition of a carrier such as water or a buffer.

The vaccine may additionally contain other immunogens for swines, such as immunogenic material characteristic of viruses such as pseudorabies virus, influenza virus, transmissible gastroenteritis virus, parvo virus, porcine endemic diarrhoea virus, hog cholera virus, or immunogenic material characteristic of mycoplasmas, such as *Mycoplasma hyopneumoniae* and *Mycoplasma lyorhinis*, or immunogenic material characteristic of bacteria, such as *Escherichia coli*, *Bordetella bronchiseptica*, *Leptospira*, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Streptococcus suis*.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows molecular size markers in kilobase pairs (kb) are given on the right hand side.

FIG. 2A is a Southern blot of Tly probe hybridized in Mutl-V strain of *Serpulina hyodysenteriae*.

FIG. 2B is a Southern blot of Kana probe hybridized in Mutl-V strain of *Serpulina hyodysenteriae*.

FIG. 2C is a Southern blot of BS probe hybridized in Mutl-V and WtC5 strain of *Serpulina hyodysenteriae*.

EXAMPLE 1

**CLONING OF A HEMOLYSIN GENE OF
*SERPULINA HYODYSENTERIAE***

Materials and Methods

Bacterial and culture conditions

Use was made of the *Serpulina hyodysenteriae* strain B204 (serotype 2) attenuated through 124 consecutive pas-

sages. The *Serpulinae* were grown in trypticase soy medium (Difco Laboratories, Detroit, Mich., USA) supplemented with 5% FCS (Flow) as described by Halter and Joens (1988; *Infect. Immun.* 56, 3152–3156). Bacterial cell pellets were washed in TE and frozen at -70°C . The plasmid pUC 19 and the phagemids pBluescript pKS+ and pSK+ (Stratagene Cloning Systems, La Jolla, Calif., USA) were utilized for the cloning procedures. *Escherichia coli* (*E. coli*) K12 strain DH5- α (Gibco BRL, Gaithersburg, Md., USA) was used as a host for these vectors.

Preparation of *Serpulina hyodysenteriae* chromosomal DNA

Molecular-grade chemicals and enzymes were from Sigma Chemical Co. (St. Louis, Mo. USA). Frozen bacterial cell pellets from 1 liter cultures were thawed in 25 ml buffer containing 100 mmol/l Tris-HCl pH 8.0, 100 mmol/l EDTA, 150 mmol/l NaCl, and 10 mg/ml lysozyme. Following a 1 hour incubation at 37°C . 0.5 ml of RNAaseA was added to the cells which were then incubated an additional 15 minutes at 70°C . Cell lysis was completed by the addition of 2.5 ml of 30% Sarkosyl, gently mixing, and incubating at 70°C . for 20 minutes followed by a 1 hour incubation at 37°C . Predigested pronase, (final concentration of 10 mg/ml) was added and incubation continued for 4 hours at 37°C . The lysate was transferred to dialysis tubing and dialyzed overnight in 6 liters of TE (10 mmol/l Tris-HCl), 1 mmol/l EDTA, pH 8.0. The DNA was then once gently extracted with TE saturated phenol, extracted with chloroform:isoamyl alcohol (24:1), dialyzed for 6 hours in TE, and ethanol precipitated. Chromosomal DNA was resuspended in TE at a concentration of 1 mg/ml. DNA prepared in this manner was used for library construction and Southern blot analysis.

Construction of *Serpulina hyodysenteriae* genomic library

Restriction enzymes, calf intestinal phosphatase, T4 DNA ligase, RNAaseA, and Klenow fragment were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind., USA). All enzymes were used under the conditions specified by the manufacturer. Standard cloning protocols (Maniatis, T. E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA) were followed for all DNA manipulations. *Serpulina hyodysenteriae* DNA was digested with the restriction enzyme MboI and ligated with T4 DNA ligase to BamHI restricted, dephosphorylated pUC19. *E. coli* DH5- α cells were transformed with the ligation mix and recombinants screened for hemolysin production.

Screening for hemolytic clones

Recombinants were plated on trypticase soy agar containing 4% defibrinated sheep red blood cells (SRBC) (Colorado Serum Co., Denver, Colo., USA) and 100 ug/ml carbenicillin (TSA blood plates). Plates were incubated at 37°C . for 24–36 hours to detect hemolytic colonies. A single hemolytic clone, designated pSML2, was chosen for further analysis. From this clone subclones were constructed.

Southern blotting

Chromosomal DNA was digested with the restriction enzyme EcoRV, electrophoresed in a 0.8% agarose gel, and transferred to a nylon membrane. A 1.5 kbp Scal/BamHI fragment from pJBA, the smallest subclone of pSML2

containing the active hemolysin gene, was random primer labeled with ^{32}P (Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specificity; *Anal. Biochem.* 132: 6–13). Prehybridization, hybridization and washing of the membrane were at 60°C . essentially as described (Maniatis, T., E. F. Fritsch and J. Sambrook. 1982. *Molecular Cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA). The membrane was exposed to Kodak X-OMAT AR film at -70°C . for periods of 2 to 18 hours.

Osmotic Release of the Recombinant Hemolysin

To characterize the recombinant hemolysin, *E. coli* DH5 α (pJBA) cells were subjected to osmotic shock essentially as described by Heppel (Heppel, L. A. 1967. Selective release of enzymes from bacteria. *Science* 156:1451–1455).

Hemolysin Assays

Aliquots of the osmotic shock supernatants were adjusted to a final concentration of 140 mmol/l NaCl and added to sheep red blood cells (SRBC) which were washed and resuspended at 10% in 140 mmol/l NaCl. The mixtures were incubated at 37°C . for one hour and the release of hemoglobin from the red cells was determined by reading the optical density of the supernatant at 540 nm.

Extraction of hemolysin from the native organism

Hemolysin was extracted from strain B204 using an RNA core extraction procedure (Kent, K. A., R. M. Lemeke, and R. J. Lyons. 1988. Production, purification and molecular weight determination of the haemolysin of *Serpulina hyodysenteriae*. *J. Mod.*

Microbiol. 27:215–224) and concentrated.

Cytotoxicity Assays

Osmotic shock supernatant from *E. coli* DH5 α (pJBA), DH5 α (pSML5) and DH5 α (pUC1 9), and RNA core-hemolysin were filter-sterilized and added to 5×10^4 Chinese Hamster Ovary (CHO) cells/well as two-fold dilutions from 1:2 to 1:160. Cells were incubated at 37°C . for 24 hours in a CO_2 incubator and examined at various time intervals for cytopathic effect (CPE). CPE was determined by direct visual inspection of the CHO monolayer at 1, 12, and 24 hours following the addition of hemolysin to each well.

DNA Sequencing

The 1.5 kbp Scal/BamHI insert in pJBA was subcloned into M13mp18 and M13mp19. Both strands were sequenced by dideoxynucleotide chain termination using a Sequenase kit (United State Biochemical, Cleveland, Ohio). The -40M13 sequencing primer was used to ascertain the sites of insertion and the first one hundred bases at the 3' and 5' regions of the gene. Subsequently, based on previous sequence, oligonucleotide primers synthesized on a Cyclone Plus DNA synthesizer (Millipore Corp., Bedford, Mass., USA) were used to sequence the complete hemolysin gene.

Results

Cloning of the hemolysin gene

The plasmid vector pUC19 was utilized to prepare a library of *Serpulina hyodysenteriae* strain B 204. Plasmid DNA from the hemolytic clone, pSML2, contained a 5 kb

fragment of *Serpulina hyodysenteriae*. The EcoR1 (E) subclone, pSML4, contained a 3.3 kb fragment and was as hemolytic as the parent plasmid pSML2. Digestion of pSML4 with Scal/BamHI(S:Scal) produced a 1.5 kb fragment which, when subcloned into EcoRV/BamHI restricted pBluescript phagemid pKS+ or pSK+, yielded the plasmid, pJBA, which was as hemolytic as either pSML2 or pSML4. The plasmid PJBA^{KS} in *E. coli* JM105 was deposited with the Centraalbureau voor Schimmelcultures at Baarn, The Netherlands under deposit number No. 512.91.

Sequence of hemolysin

The hemolysin gene was exceptionally adenosine-plus-thymidine rich (75%) as has been reported for pathogenic and non-pathogenic strains of Serpulinas (Miao, R. M., A. H. Fieldsteel, and D. L. Harris. 1970. Genetics of *Treponema*: characterization of *Treponema hyodysenteriae* and its relationship to *Treponema pallidum*. Infect. Immun. 22: 736–739). The sequence is shown in SEQUENCE ID NO. 1.

EXAMPLE 2

PREPARATION OF A *SERPULINA HYODYSENTERIAE* INSERTION MUTANT

MATERIALS AND METHODS

Bacterial strains and plasmid

Serpulina hyodysenteriae C5 (deposited on 18 Dec. 1991 at the Centraalbureau voor Schimmelcultures at Baarn, the Netherlands under deposit number CBS 837.91) is cultured under the same conditions as described in Example 1 for the strain B204. The vector pBluescript 11 KS(+) was purchased from Stratagene (La Jolla, Calif.) and grown in *E. coli* K12 DH5 α . Mice cecal contents were plated on media as described before.

Construction of a hemolysin gene containing a kanamycin resistance gene

For the construction of a hemolysin negative mutant use was made of plasmid PJBA^{KS}, containing the 1.5 kb Scal/BamHI fragment including the hemolysin gene (further indicated as tly) of *Serpulina hyodysenteriae* B204 with a unique BglII site. A 1.3 kb Kanamycin Resistance GenBlock (EcoR1) (Pharmacia) was digested with the restriction enzyme BamHI and was inserted into this BglII site. The resulting PJBA^{KS} derivative was named pTly-.

Electroporation

Serpulina hyodysenteriae C5 was grown in 200 ml trypticase soy broth (TSB), supplemented with 5% fetal calf serum and 0.05% RNA core, for 48 hours at 42° C. under anaerobic conditions. Cells were centrifuged, washed and harvested in 50 ml of icecold 15% glycerol-272 mM sucrose, centrifuged and resuspended to 10¹⁰ cells/ml in the same medium. Aliquots were frozen and kept at -80° C. until used. 50 μ l of cell suspension was mixed with 5 μ g of DNA in water. Electroporation was performed with a Bio-Rad Gene Pulser with pulse controller in 0.56 mm gap cuvettes (Biotechnologies and Experimental Research Inc., San Diego, Calif.) at 0.6kV, 25 μ F and 200 Ω . This leads to time constants ranging from 3.5 to 4 ms. Cells were recovered in 1 ml TSB and poured onto trypticase soy agarplates, supplemented with 5% sheep blood and 400 μ g of spectinomycin per ml (TSAB+). After 8 and 14 h of regeneration at 42° C. under anaerobic conditions, cells were harvested,

plated onto TSAB+ agar plates supplemented with 30 or 150 μ g/ml kanamycin and grown for 4 days at 42° C. under anaerobic conditions. Colonies were screened for diminished hemolysis. The electroporation experiments were carried out in duplicate.

Polymerase chain reaction

Colonies with diminished hemolysis detected after electroporation, and wildtype (Wt) *Serpulina hyodysenteriae* C5 were screened with polymerase chain reaction (PCR). DNA amplification with Taq polymerase and Taq polymerase buffer (Promega) was performed with primers corresponding to nucleotides 471–486 and 1449–1431 of the pJBA Sequence ID No. 1. This resulted in an amplified product of 0.98 kb of the tly gene including the BglII site. For the Wt C5, 0.5 μ g DNA was used. Colonies of the mutants were touched with a sterile toothpick and transferred to the PCR buffer. After the mixtures were preheated at 95° C. for 3 minutes, 35 amplification cycles were performed in a DNA Incubator Prem, as follows: 1 min at 95° C., 1 min at 40° C. and 2 min at 72° C. After the final cycle, the mixture was incubated for 10 min at 72° C. to complete the last polymerase reaction. Reaction mixtures were analyzed by agarose gel electrophoresis as described before.

Isolation of DNA and Southern blot analysis

Plasmid DNA was isolated from *E. coli* and *Serpulina hyodysenteriae* by the method of Birnboim and Doly (Nucleic Acids Research 7, 1513–1523; 1989). Chromosomal DNA of *Serpulina hyodysenteriae* was isolated by isopropanol precipitation and phenol extraction. DNA was digested with EcoRV as indicated by the supplier (BRL). EcoRI digests of pBluescript II KS(+) and a pBluescript II KS(+)/Kanamycin Resistance Genblock construct were taken as controls. Hybridization on Hybond N (Amersham) and washing was performed as described before. The 1.5 kb Scal/BamHI fragment of PJBA^{KS} (Tly probe), the 1.3 kb Kanamycin Resistance Genblock (Pharmacia) (Kana probe) and the complete vector Bluescript (BS probe) were radiolabeled and used as probes.

Virulence test in mice

The mouse challenge studies were done as described before. Six week old female OF-1 mice (Iffa Credo) were kept under controlled conditions. Feed was removed 14 h before the first challenge and was withheld for 40 hours. Mice were administered two consecutive doses (24 h apart) by gastric intubation with 0.5 ml of a log-phase culture of serpulinas: 10⁶ or 10⁸ CFU *Serpulina hyodysenteriae* Wt C5, 10⁶ or 10⁸ CFU *Serpulina hyodysenteriae* C5 tly-, or 10⁸ CFU *Serpulina innocens* ATCC 29796. Three control mice were inoculated with 0.5 ml TSB. The mice were necropsied 12 days later to evaluate signs of *Serpulina hyodysenteriae* infection in the cecum: catarrhal inflammation, excess intraluminal mucus, oedema, hyperemia and atrophy. Cecal contents were cultured to assay shedding of serpulinas.

Results

Electroporation

In two separate experiments with the pTly construct, a total of 5 kanamycin resistant colonies with diminished hemolysis were recovered. In the first experiment, when cells were plated on TSAB+ plates with only 30 μ g/ml kanamycin, one less hemolytic colony (MuII) was recovered

among many strong hemolytic colonies. In the second experiment, when cells were plated on TSAB+ plates with 150 µg/ml kanamycin, 4 colonies with diminished hemolysis (MutII-V) were found.

Polymerase chain reaction

Upon analysis of the PCR products of *Serpulina hyodysenteriae* Wt C5 and MutI-MutV by agarose gel electrophoresis, Wt C5 and MutIV showed a fragment of only 0.98 kb, which is the expected size of the fragment of the tly gene amplified by the primers of pJBA used. MutI, MutII, MutII and MutV showed a fragment of 2.28 kb (0.98 kb of the tly gene and 1.30 kb of the kanamycin gene block) (FIG. 1; molecular size markers in kilobase pairs (kb) are given on the right hand side of this figure).

DNA isolation and Southern blot analysis

No plasmid DNA could be isolated from the mutants MutI-V. Chromosomal DNA of *Serpulina hyodysenteriae* Wt C5 and MutI-V was digested with EcoRV, blotted and hybridized with the Tly probe, Kana probe and BS probe respectively. In strain Wt C5 the Tly probe hybridized with a fragment of 4.8 kb. In MutI this probe hybridized with a fragment of 6.1 kb (i.e. 4.8 kb plus 1.3 kb kanamycin gene insertion) (FIG. 2A). Strain Wt C5 did not hybridize with the Kana probe. A fragment of 6.1 kb in MutI hybridized with the Kana probe (FIG. 2B). Neither MutI nor WtC5 hybridized with the BS probe (FIG. 2C).

Virulence test of MutI in Mice

Six groups of OF-1 mice were challenged with 10⁶ or 10⁸ CFU of *Serpulina hyodysenteriae* Wt C5, 10⁶ or 10⁸ CFU of MutI, 10⁸ CFU of *Serpulina innocens* ATCC 29796, or TSB (controls). Mice were killed at day 12 for evaluation of cecal lesions (catarrhal inflammation, excess intraluminal mucus, oedema, hyperemia and atrophy) and colonization by ser-

pulinas. Cecal scores are represented in Table 1. Macroscopic cecal lesions were scored as follows: severe lesions, 3; moderate lesions, 2; mild lesions, 1; no lesions, 0. Macroscopic cecal lesions were less severe in mice infected with MutI (both inoculation doses) than in mice infected with *Serpulina hyodysenteriae* Wt C5. Mice infected with *Serpulina innocens* or inoculated with TSB, did not show any cecal lesions. The number of mice that were culture positive, are also shown in Table 1. In the group infected with *Serpulina innocens*, no mouse was culture positive.

TABLE 1

group mice ^e	CFU	n ^f	group mean cecal score	number of mice lesion positive	number of mice culture positive
Wt C5	10 ⁸	7	2.42	7	7
Wt C5	10 ⁶	6	1.66	6	5
MutI	10 ⁸	7	1.28	5	7
MutI	10 ⁶	7	1.00	5	4
S.inno ^c	10 ⁸	7	0	0	0
TSB		3	0	0	0

^a*Serpulina hyodysenteriae* C5 wildtype

^bMutI = hemolysin tly-mutant of *Serpulina hyodysenteriae* C5

^cS.inno = *Serpulina innocens* American Type culture Collection (ATCC) 29796

^dTSB = trypticase soy broth

^efemale SPF OF-1 mice (Iffa Credo)

^fn = number of mice per group

^gcecal score: macroscopic cecal lesions were scored as follows: severe lesions, 3; moderate lesions, 2; mild lesions, 1; no lesions, 0.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 3

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1498 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(v i) ORIGINAL SOURCE:

- (A) ORGANISM: SERPULINA HYODYSENTERIAE
- (B) STRAIN: B 204
- (H) CELL LINE: E. COLI JM105 (pJBA) [CBS 512.91]

(i x) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..456
- (D) OTHER INFORMATION: /product= "UNKNOWN PROTEIN"

(i x) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 457..470

-continued

(i x) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 471..1190

(D) OTHER INFORMATION: /product= "HEMOLYSIN PROTEIN"

(i x) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1191..1498

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAT	CCT	AAT	GCT	GAT	ACT	GAT	GAA	TCT	CCT	GCT	TTA	TTG	ATT	TCT	GCT	48
Asp	Pro	Asn	Ala	Asp	Thr	Asp	Glu	Ser	Pro	Ala	Leu	Leu	Ile	Ser	Ala	
1				5			10							15		
TCT	ATA	ACT	GAT	ACT	GAT	ACA	GTT	AAA	GTA	ATA	TTA	CAG	GCA	TTT	GCT	96
Ser	Ile	Thr	Asp	Thr	Asp	Thr	Val	Lys	Val	Ile	Leu	Gln	Ala	Phe	Ala	
			20				25						30			
GAA	GAT	GTT	ACT	GAT	GAT	ATT	TAT	ACA	ATT	GGC	GGT	AAT	TTA	TGC	TAT	144
Glu	Asp	Val	Thr	Asp	Asp	Ile	Tyr	Thr	Ile	Gly	Gly	Asn	Leu	Cys	Tyr	
		35				40						45				
ATA	AAA	GAT	TCT	ATA	TTA	TAT	ATT	TCT	GAT	AAT	TCT	AAT	GTT	ATA	GAT	192
Ile	Lys	Asp	Ser	Ile	Leu	Tyr	Ile	Ser	Asp	Asn	Ser	Asn	Val	Ile	Asp	
	50					55					60					
TCT	ATA	ATT	AAT	GGT	GAA	AAG	CCA	GCA	ACA	GCA	TTA	TCT	GCT	GAT	AAA	240
Ser	Ile	Ile	Asn	Gly	Glu	Lys	Pro	Ala	Thr	Ala	Leu	Ser	Ala	Asp	Lys	
				70						75					80	
GTT	GAA	ATA	GCT	AAA	AAT	AAT	ACT	ATG	GCT	TTA	TAT	TTA	GAG	TTT	AAT	288
Val	Glu	Ile	Ala	Lys	Asn	Asn	Thr	Met	Ala	Leu	Tyr	Leu	Glu	Phe	Asn	
				85					90					95		
TCT	AAT	TTA	TCA	TTA	TAT	GGT	ATT	GGA	GAT	GAA	TAT	ACT	GAA	ACT	TTT	336
Ser	Asn	Leu	Ser	Leu	Tyr	Gly	Ile	Gly	Asp	Glu	Tyr	Thr	Glu	Thr	Phe	
			100					105					110			
GAA	TCA	GTT	TAT	ATA	ACT	TCA	AAT	ATA	TTA	GAA	AGC	AAT	CAT	ACT	CAA	384
Glu	Ser	Val	Tyr	Ile	Thr	Ser	Asn	Ile	Leu	Glu	Ser	Asn	His	Thr	Gln	
		115					120					125				
ATG	CTT	TTA	AAA	GTA	AAT	ATG	AGA	GAT	AAA	GAA	AGA	AAT	TCT	CTT	TCT	432
Met	Leu	Leu	Lys	Val	Asn	Met	Arg	Asp	Lys	Glu	Arg	Asn	Ser	Leu	Ser	
	130					135						140				
ATA	ATA	AAA	TCT	TTC	CTT	GGA	TTA	TAATACTAAT	ATAAA	ATG	CGA	TTA	GAT			482
Ile	Ile	Lys	Ser	Phe	Leu	Gly	Leu			Met	Arg	Leu	Asp			
					150					1						
GAA	TAT	GTG	CAT	AGT	GAA	GGC	TAT	ACA	GAA	AGC	AGA	TCT	AAA	GCA	CAG	530
Glu	Tyr	Val	His	Ser	Glu	Gly	Tyr	Thr	Glu	Ser	Arg	Ser	Lys	Ala	Gln	
	5				10					15					20	
GAT	ATA	ATA	CTA	GCC	GGT	TGT	GTT	TTT	GTT	AAT	GGA	GTA	AAG	GTA	ACT	578
Asp	Ile	Ile	Leu	Ala	Gly	Cys	Val	Phe	Val	Asn	Gly	Val	Lys	Val	Thr	
				25					30					35		
TCT	AAG	GCT	CAT	AAA	ATA	AAA	GAT	ACT	GAT	AAT	ATA	GAA	GTT	GTT	CAG	626
Ser	Lys	Ala	His	Lys	Ile	Lys	Asp	Thr	Asp	Asn	Ile	Glu	Val	Val	Gln	
			40					45					50			
AAT	ATA	AAA	TAT	GTA	TCA	AGA	GCT	GGA	GAA	AAA	TTA	GAA	AAG	GCG	TTT	674
Asn	Ile	Lys	Tyr	Val	Ser	Arg	Ala	Gly	Glu	Lys	Leu	Glu	Lys	Ala	Phe	
		55					60					65				
GTA	GAA	TTT	GGA	ATA	TCT	GTA	GAA	AAT	AAA	ATA	TGT	TTA	GAT	ATA	GGA	722
Val	Glu	Phe	Gly	Ile	Ser	Val	Glu	Asn	Lys	Ile	Cys	Leu	Asp	Ile	Gly	
	70					75					80					
GCT	TCT	ACA	GGA	GGA	TTT	ACA	GAT	TGT	CGT	CTT	AAG	CAT	GGT	GCT	AAA	770
Ala	Ser	Thr	Gly	Gly	Phe	Thr	Asp	Cys	Arg	Leu	Lys	His	Gly	Ala	Lys	
	85				90					95					100	
AAA	GTT	TAT	GCT	CTT	GAT	GTA	GGA	CAT	AAT	CAG	CTA	GTT	TAT	AAA	CTT	818
Lys	Val	Tyr	Ala	Leu	Asp	Val	Gly	His	Asn	Gln	Leu	Val	Tyr	Lys	Leu	
				105					110					115		
CGT	AAT	GAT	AAT	AGG	GTA	GTG	TCA	ATA	GAA	GAT	TTC	AAT	GCC	AAA	GAT	866

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Arg	Asn	Asp	Asn	Arg	Val	Val	Ser	Ile	Glu	Asp	Phe	Asn	Ala	Lys	Asp	
			120					125					130			
ATA	AAT	AAA	GAA	ATG	TTC	AAT	GAT	GAA	ATC	CCA	TCT	GTA	ATA	GTA	AGT	914
Ile	Asn	Lys	Glu	Met	Phe	Asn	Asp	Glu	Ile	Pro	Ser	Val	Ile	Val	Ser	
		135					140					145				
GAC	GTA	TCA	TTT	ATA	TCA	ATA	ACA	AAA	ATA	GCA	CCA	ATC	ATA	TTT	AAA	962
Asp	Val	Ser	Phe	Ile	Ser	Ile	Thr	Lys	Ile	Ala	Pro	Ile	Ile	Phe	Lys	
	150					155					160					
GAA	TTA	AAT	AAT	TTA	GAG	TTT	TGG	GTA	ACT	TTA	ATA	AAA	CCA	CAA	TTT	1010
Glu	Leu	Asn	Asn	Leu	Glu	Phe	Trp	Val	Thr	Leu	Ile	Lys	Pro	Gln	Phe	
165					170				175						180	
GAA	GCT	GAA	AGA	GGT	GAT	GTT	TCA	AAA	GGC	GGT	ATA	ATA	CGA	GAT	GAT	1058
Glu	Ala	Glu	Arg	Gly	Asp	Val	Ser	Lys	Gly	Gly	Ile	Ile	Arg	Asp	Asp	
				185					190					195		
ATA	CTT	AGA	GAA	AAA	ATA	TTA	AAT	AAT	GCT	ATT	TCA	AAG	ATA	ATA	GAC	1106
Ile	Leu	Arg	Glu	Lys	Ile	Leu	Asn	Asn	Ala	Ile	Ser	Lys	Ile	Ile	Asp	
			200				205						210			
TGC	GGA	TTT	AAA	GAA	GTT	AAT	AGA	ACC	ATC	TCT	CCT	ATA	AAA	GGT	GCT	1154
Cys	Gly	Phe	Lys	Glu	Val	Asn	Arg	Thr	Ile	Ser	Pro	Ile	Lys	Gly	Ala	
		215					220					225				
AAA	GGT	AAT	ATA	GAA	TAT	TTA	GCT	CAT	TTT	ATT	ATT	TAATCATT	TTT			1200
Lys	Gly	Asn	Ile	Glu	Tyr	Leu	Ala	His	Phe	Ile	Ile					
	230					235					240					
CTATTTTATG	TGTATTTCTC	TGTTTATATA	TTTCATATTC	TTTATAGAAG	CCTTCTACAT											1260
CATTTACCAT	TAAATATCCT	TCTTCTGATA	TATCTAATGA	TTTTATTTTT	AATATTTTCAT											1320
TTTCTACATT	ACTTTTATAT	TCTATGCCTA	TCATAGAACA	AATATCATT	ATATTATATT											1380
GAAATTTTAT	TTTGTTTATA	TTTTTGAATA	AAAGTTCAGT	TTTTATTAAC	GCTTCTATTA											1440
TTATCACGAA	TTTGCTTACT	ACTTTATTAG	CATTAAAAGA	CCTTATTCTA	GAAATAGT											1498

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 152 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asp	Pro	Asn	Ala	Asp	Thr	Asp	Glu	Ser	Pro	Ala	Leu	Leu	Ile	Ser	Ala	
1				5					10					15		
Ser	Ile	Thr	Asp	Thr	Asp	Thr	Val	Lys	Val	Ile	Leu	Gln	Ala	Phe	Ala	
			20					25					30			
Glu	Asp	Val	Thr	Asp	Asp	Ile	Tyr	Thr	Ile	Gly	Gly	Asn	Leu	Cys	Tyr	
		35					40					45				
Ile	Lys	Asp	Ser	Ile	Leu	Tyr	Ile	Ser	Asp	Asn	Ser	Asn	Val	Ile	Asp	
	50					55					60					
Ser	Ile	Ile	Asn	Gly	Glu	Lys	Pro	Ala	Thr	Ala	Leu	Ser	Ala	Asp	Lys	
	65				70					75					80	
Val	Glu	Ile	Ala	Lys	Asn	Asn	Thr	Met	Ala	Leu	Tyr	Leu	Glu	Phe	Asn	
				85					90					95		
Ser	Asn	Leu	Ser	Leu	Tyr	Gly	Ile	Gly	Asp	Glu	Tyr	Thr	Glu	Thr	Phe	
			100					105					110			
Glu	Ser	Val	Tyr	Ile	Thr	Ser	Asn	Ile	Leu	Glu	Ser	Asn	His	Thr	Gln	
		115					120					125				
Met	Leu	Leu	Lys	Val	Asn	Met	Arg	Asp	Lys	Glu	Arg	Asn	Ser	Leu	Ser	
	130					135						140				

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I l e I l e L y s S e r P h e L e u G l y L e u
1 4 5                1 5 0
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(2) INFORMATION FOR SEQ ID NO:3:

```
( i ) SEQUENCE CHARACTERISTICS:
      ( A ) LENGTH: 240 amino acids
      ( B ) TYPE: amino acid
      ( D ) TOPOLOGY: linear
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(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met  Arg  Leu  Asp  Glu  Tyr  Val  His  Ser  Glu  Gly  Tyr  Thr  Glu  Ser  Arg
  1      5      10      15
Ser  Lys  Ala  Gln  Asp  Ile  Ile  Leu  Ala  Gly  Cys  Val  Phe  Val  Asn  Gly
      20      25      30
Val  Lys  Val  Thr  Ser  Lys  Ala  His  Lys  Ile  Lys  Asp  Thr  Asp  Asn  Ile
      35      40      45
Glu  Val  Val  Gln  Asn  Ile  Lys  Tyr  Val  Ser  Arg  Ala  Gly  Glu  Lys  Leu
      50      55      60
Glu  Lys  Ala  Phe  Val  Glu  Phe  Gly  Ile  Ser  Val  Glu  Asn  Lys  Ile  Cys
      65      70      75      80
Leu  Asp  Ile  Gly  Ala  Ser  Thr  Gly  Gly  Phe  Thr  Asp  Cys  Arg  Leu  Lys
      85      90      95
His  Gly  Ala  Lys  Lys  Val  Tyr  Ala  Leu  Asp  Val  Gly  His  Asn  Gln  Leu
      100     105     110
Val  Tyr  Lys  Leu  Arg  Asn  Asp  Asn  Arg  Val  Val  Ser  Ile  Glu  Asp  Phe
      115     120     125
Asn  Ala  Lys  Asp  Ile  Asn  Lys  Glu  Met  Phe  Asn  Asp  Glu  Ile  Pro  Ser
      130     135     140
Val  Ile  Val  Ser  Asp  Val  Ser  Phe  Ile  Ser  Ile  Thr  Lys  Ile  Ala  Pro
      145     150     155     160
Ile  Ile  Phe  Lys  Glu  Leu  Asn  Asn  Leu  Glu  Phe  Trp  Val  Thr  Leu  Ile
      165     170     175
Lys  Pro  Gln  Phe  Glu  Ala  Glu  Arg  Gly  Asp  Val  Ser  Lys  Gly  Gly  Ile
      180     185     190
Ile  Arg  Asp  Asp  Ile  Leu  Arg  Glu  Lys  Ile  Leu  Asn  Asn  Ala  Ile  Ser
      195     200     205
Lys  Ile  Ile  Asp  Cys  Gly  Phe  Lys  Glu  Val  Asn  Arg  Thr  Ile  Ser  Pro
      210     215     220
Ile  Lys  Gly  Ala  Lys  Gly  Asn  Ile  Glu  Tyr  Leu  Ala  His  Phe  Ile  Ile
      225     230     235     240
```

We claim:

1. A mutant strain of *Serpulina hyodysenteriae* capable of eliciting a protective immune response against a wild-type virulent strain of *Serpulina hyodysenteriae* which mutant strain is less virulent than the wild-type strain, wherein the expression of hemolysin encoded by the chromosomal tly gene is abolished through the deletion of the entire tly gene.

2. A mutant strain of *Serpulina hyodysenteriae* capable of eliciting a protective immune response against a wild-type virulent strain of *Serpulina hyodysenteriae* which mutant strain is less virulent than the wild-type strain, said mutant strain being generated by using a genetic engineering technique comprising introducing an insertion mutation into the

tly gene that results in a shift in the reading frame of the tly gene, wherein said insertion mutation is a polynucleotide coding for a selectable characteristic whereby the expression of hemolysin encoded by the chromosomal tly gene is abolished.

3. A mutant strain according to claim 2, wherein said polynucleotide is inserted in the BglIII site located between nucleotides 506–511 of SEQ ID NO: 1.

4. A vaccine containing a mutant strain of *Serpulina hyodysenteriae* according to claim 1 and a suitable carrier.

5. A vaccine containing a mutant strain of *Serpulina hyodysenteriae* according to claim 2 and a suitable carrier.

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6. A vaccine containing a mutant strain of *Serpulina hyodysenteriae* according to claim 3 and a suitable carrier.

7. A method for reducing the severity of a *Serpulina hyodysenteriae* infection, comprising administering to a susceptible host a vaccine according to claim 4. 5

8. A method for reducing the severity of a *Serpulina hyodysenteriae* infection, comprising administering to a susceptible host a vaccine according to claim 5.

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9. A method for reducing the severity of a *Serpulina hyodysenteriae* infection, comprising administering to a susceptible host a vaccine according to claim 6.

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